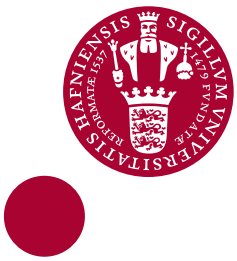

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Find, Jens Iver

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Towards industrial production of tree varieties through somatic embryogenesis and other vegetative propagation technologies: Nordmanns fir (*Abies nordmanniana* (Steven) Spach) - From research laboratory to production

J. I. Find

Tissue Culture Laboratory, Institute of Geosciences and Natural Ressources, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark.
e-mail : jensf@ign.ku.dk

Abstract

The main focus of the research on somatic embryogenesis in nordmanns fir has until recently been on improving the basic protocols in each step of the process. However, with recent developments, one single set of methods has shown to be effective for production of plants from more than 500 different untested cell lines. The developed method ensured a very good selection of genotypes at any of the involved steps: initiation, proliferation, cryo preservation, maturation, germination, and nursery culture. Growth and development of clonally propagated plants in the field were similar to those of seed produced plants. For this reason the focus is changing towards improvement of protocols for cost effective large scale production in a commercial set up. The first elite clones have been identified from a small preliminary clonal field trial from 2007, and larger clonal field trials have been established in 2014 and 2015 with 9000 plants from 400 clones.

Keywords: Christmas trees, clonal propagation, automation, field tests, elite trees, somatic embryogenesis

1. Introduction

Nordmanns fir or Caucasian fir (*Abies nordmanniana* (Steven) Spach) originates from the Caucasus Mountains and the Northern Turkey. The species is not widely used for traditional forest products such as timber or pulp and paper. The commercial interest is primarily focussed on the production of Christmas trees

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and greenery. Nordmanns fir is grown as plantation forestry in Denmark and in several other countries in Northern Europe and in the USA. The Christmas tree industry is of steadily growing importance and nordmanns fir has become the economically most important tree species in Danish forestry. The European market is growing from the present state of approximately 50 million trees each year.

The production of Christmas trees is a typical example of agroforestry and it has many similarities with the production of more traditional high value agricultural crops. Christmas trees are grown in mono cultures and the rotation time of 8-10 years is very short compared to traditional forestry. The production is cost and labour intensive, and the single plant represents a sizeable value for the grower.

Propagation of Christmas trees is exclusively from seeds collected in the natural forests in the Caucasus or from Danish seed plantations. The seed supply is unstable and genetic variability is prominent. Nordmanns fir has a generation time of 25-30 years, and traditional breeding programmes are extremely time consuming. The extended generation period is a general problem in breeding programmes for forest trees, but it is particularly a problem in specialized industries, dependent on fast breeding and development of new products.

Even with intensive management and shaping of trees, the growers must expect a loss of 25-35 % of trees due to low quality, when trees are propagated from seeds. Only about 10-15 % of the produced trees are of best quality (Table 1). Clonal propagation of elite material offers the ideal propagation method, with high and uniform quality of trees. The expected gain by use of clonal propagation is estimated to be approximately 3.6 Euro per tree, compared to the present situation with seed propagated material (Table 1). This gain is based on an average better quality of trees. In addition to this, the uniformity in itself provides a predictable production focussed on the specific clone and the possibility of clearcutting the area in one year instead of over several years because of the uniformity of the trees and thus shortening the production time with one or two years.

The application of mono cultures in combination with the 8-10 year rotation time increases the risk of damage caused by insect predation. This is a considerable problem in the Christmas tree production where the form and appearance is of major importance for the final quality and value of the product. At present the problem is mainly met by application of pesticides. However, the public acceptance of this solution is declining, and selection of clones with natural resistance will be the best way of preventing serious damage caused by insects.

Infection by the fungus *neonectria neomacrocarpa* has lately caused severe damage in Danish plantations. The biology behind the infection is not known and the only way of dealing with the problem is to remove infected trees. In 2013, the fungus caused an estimated economic loss of almost 10 million Euros in Denmark, and in future this fungus can cause serious economic losses for the

Table 1. *Nordmanns fir. An example of economic gain by integration of clonal propagation techniques in breeding programs of forest trees. Distribution of trees in categories of quality and estimated sales price when propagated from seedlings or by clonal propagation of elite material (Find et al. 2009). Calculation of average sales price per 100 trees. Estimated numbers were obtained from the Danish Christmas tree Growers Association*

Gain for the grower by use of cloned material					
	%-distribution		Price per tree	Sales price per 100 trees	
Categories, quality	Seeds	Cloning	(Euro)	Seeds	Cloning
Excellent	15	60	12.3	185	738
Standard	40	20	6.9	276	138
Below standard	20	5	3.7	74	185
Rejects on basis of form	20	10	0	0	0
Rejects on basis of damage	5	5	0	0	0
Total	100	100		535	895
Expected gain per tree by clonal propagation: 3.6 Euro					

industry. Resistance towards the fungus seems to have a genetic background, and it is expected that it is possible to select resistant clones (Thomsen et al. 2014). Clonal propagation of resistant trees may be the only protection against serious threats from this and other fungi.

Traditional methods for clonal propagation, such as cuttings, are not possible for nordmanns fir because of a very low rooting rate and plagiotropic growth. Somatic embryogenesis (SE) is at present the only promising method for clonal propagation in nordmanns fir, and the method offers great potential for enhancing gains from intensive Danish tree-breeding programs and for bulk propagation of identified elite trees. Establishment of SE in nordmanns fir was reported for the first time in 1991 (Nørgaard and Krogstrup 1991). Since then the protocols have been further developed (Nørgaard and Krogstrup 1995, Nørgaard 1997 and Find et al. 2002) including development of standard methods for cryopreservation (Nørgaard et al. 1993) and for genetic transformation (Find et al. 2005).

The SE system is developed to a state where it is ready to be tested in a commercial set up. The aim of this chapter is to present the state of the art for this species in our laboratory, and to outline how the technologies are transferred from a research laboratory to a production facility in a commercial set up.

2. Methods in relation to large scale production

2.1 Induction of embryogenic tissue

Embryogenic tissue can be initiated from immature zygotic embryos (Nørgaard and Krogstrup 1991) and from mature zygotic embryos; from fresh or stored and dried seeds (Nørgaard and Krogstrup 1995, Kristensen et al. 2005). Induction of SE is possible all year round. SE is initiated from the hypocotyl after 8-12 weeks of culture (Nørgaard and Krogstrup 1995). The frequency of initiation is very dependent on the quality of seeds. From fresh seeds, initiation rates are from 50-85 % (Kristensen et al. 2005). This rate is large enough to allow setting up clonal tests from selected families or controlled crossings. When the embryogenic tissue has a diameter of approximately 0.5-1.0 cm it is removed from the explant and transferred to fresh medium for proliferation (Figure 1).

Figure 1. *Vegetative propagation of nordmanns fir by somatic embryogenesis.* ***A.*** *Cell culture initiated from post cotyledonary embryos isolated from stored seeds.* ***B.*** *Mature embryos after 12 weeks of maturation.* ***C.*** *Germinated embryos in a petri dish.* ***D.*** *Plants after one growth season in a plug system.* ***E.*** *Plants in the greenhouse. Two years old and ready for transfer to clonal field testing in the fall of 2014. Photo May 2014.* ***F.*** *Three plants from the same clone in the second growth season, May 2014. (Photos E Bihrmann).*

2.2 Maintenance of embryogenic tissue

The embryogenic cultures are maintained on solid proliferation medium by subculture of tissue to fresh medium every 2 weeks. Proliferation of somatic

embryos in nordmanns fir is by continuous cleavage of embryos (Figure 1), and cultures double in size every two weeks. Embryogenic potential will be maintained over many years on solid proliferation medium, but requirements of the maturation medium may change during prolonged periods of proliferation (Find et al. 2002).

2.3 Cryopreservation

All cell lines are stored in liquid nitrogen (Nørgaard and Krogstrup 1995). At present the laboratory holds a gene bank of approximately 900 frozen cell lines of nordmanns fir. The cell lines originated from the Danish breeding program or from selected trees in the natural stands in Georgia.

2.4 Maturation

As opposed to many other conifer SE systems, addition of the growth regulator abscisic acid (ABA) alone does not induce development of high quality cotyledonary embryos in cell lines of nordmanns fir. To increase the number and the quality of mature embryos it is necessary to include an additional step where the auxin antagonist PCIB is added (Find 2001, Find et al. 2002). In a recent test of 400 cell lines from 40 families, high quality mature embryos were produced from 98 % of the cell lines with use of only one standard method including PCIB treatment (unpublished results). Mature embryos are harvested after 12-14 weeks, and a second harvest is possible one week later. Only high quality embryos with no observed abnormalities are harvested. In our experience any type of irregularity in the outer appearance or shape of mature embryos leads to improper development and low performance during germination and plant production (unpublished results). For larger scale production of plants, optimisation of the protocol for each specific cell line will be essential. This will increase the total number of embryos, the average quality of embryos and will importantly improve synchronisation of the development of embryos from the same batch. At present maturation is set up on filter paper on solid medium in 10 cm Petri dishes (Figure 1) (Find et al. 2002). This method is effective for production of a few hundred mature embryos from each of a large number of clones needed for the present establishment of clonal trials. Cheaper and more efficient methods are essential for large scale production from a few selected clones.

2.5 Germination and transfer to soil

Different ways of ‘after ripening’ or pre-treatments have been developed to improve conversion and germination of mature somatic embryos of nordmanns fir. In our experience, the main parameter for successful plant production is the quality

of the mature embryo. Previously, transfer from sterile conditions to soil in the nursery was the main bottleneck for practical application of SE in nordmanns fir. However, lately this has changed and now the growth of clonally propagated plants in the greenhouse is comparable or better than that of similar plants produced from seeds (unpublished results). To reduce production costs, it was tested how early in the process plants can be transferred to the production green house without compromising survival and growth. Interestingly, preliminary tests have shown improved growth of plants in the greenhouse compared to the growth obtained presently in ventilated plastic boxes in the controlled growth room (unpublished results). The reason may be better airflow and increased control of physical factors in a larger scale production facility.

3. Clonal field trials

The establishment of clonal field trials has high priority, as they serve as the basis for the future selection of elite material for establishment of a commercial production. The primary criteria for selection in the Christmas tree production will be the general appearance of trees. Growth rate, form and color are important factors, but the final selection will, to some extent, be based on personal preferences regarding tree form. Additionally, quantitative parameters such as: needle retention, frost tolerance, pest and fungi resistance are very important parameters. Natural resistance towards insects and fungi may increase in importance, because consumer demands for 'natural trees' that are grown without the use of pesticides and fungicides are increasing. Screening for some parameters is possible after a few years of growth, but the final selection may not be possible until a full growth period of 8-10 years has passed.

Figure 2. *Clonal field trial with nordmanns fir established in 2007. After 8 growth seasons. (Photo E. Bihrmann, 2015).*

Our first clonal trial with SE plants of nordmanns fir was established in the fall of 2007 with 379 trees from 9 clones (Find 2014). The aim of this field trial was not primarily to select elite material, but to investigate the growth of SE plants compared to seedlings and to document the phenotypic uniformity of ramets from the same clone. These plants are now eight growth seasons old (Fig 2). The nine clones were phenotypically very different from each other, as one would expect for randomly selected trees with different genetic backgrounds. Opposite to this, phenotypical variation in growth, form, color and time of sprouting was very small between ramets from same clone. Two clones out of the nine clones in total may have commercial potential. One of these clones was fast growing and form and appearance was superior to that of the average for Christmas trees (Fig 3).

Another clone was very slow growing and the appearance was dense and compact (Fig 4). This clone is not suited for normal production of Christmas trees, but has gained interest from producers of potted trees. In 2011, 47 mother trees/families were selected from natural stands in Georgia. Cones were collected

Figure 3 (Left). *Clone selected in 2015 from a field trial established in 2007. The clone is fast growing, and form and appearance is superior to that of the average for Christmas trees. (Photo E. Bihrmann, 2015).*

Figure 4 (Right). *Clone selected in 2015 from a field trial established in 2007. This clone is not suited for normal production of Christmas trees, but has due to its low and compact appearance gained interest from producers of potted trees. Notice the uniform phenotypic appearance of all trees in the row. (Photo E. Bihrmann, 2015).*

from all selected trees and SE was established from 250 cell lines. In 2012, an additional 27 trees were selected from the Danish breeding program. SE was established from 150 cell lines. All clones/cell lines were stored in the cryogenic gene bank.

In the fall of 2014 approximately 4.000 plants at an age of two years, originating from the 250 clones established in 2011, were planted in clonal field trials situated at two different locations in Denmark. All clones were planted on both locations to investigate environmental impact on growth and development. The field trials were randomized and mixed with seedlings of the same age. After one year of growth in the field, survival rate was 90-95 %, and the growth was comparable to the growth of seedlings (unpublished results). Similar field trials with approximately 5.000 plants from an additional 150 clones will be established during the fall of 2015.

4. From research to production

Somatic embryogenesis in nordmanns fir has shown to be effective in the research laboratory and growth of the produced plants is not different from that of seedlings. The next step is to test the methods in commercial scale production. There are many advantages in going from production of a few plants from each of several hundred clones, as in production of plants for clonal field testing, to production of commercial scale amounts of plants from a few selected clones in a production line. In large scale production of a few clones, it is possible to gain experience of the biology of each particular clone/cell line, and each step in the production can be optimized and scheduled to fit to the particular clone/cell line.

There are still unknown biological aspects in SE, which need attention in setting up of a commercial production. The embryogenic cultures of nordmanns fir have shown to be very stable over time in respect to their maturation capacity. This may change during prolonged periods of scale up and large scale production. In order to produce uniform plants and to reduce production costs, developmental steps such as maturation, rooting, and shoot growth must be synchronized in the production. This is not always the case in the present set up, and this must be considered in a larger scale set up. To achieve a cost effective and uniform production, the nursery must have plants delivered during a short period of, e.g., one month each year. To take advantage of the fact that plants can be produced in the laboratory all year, it is necessary to find effective means of arresting the development of mature somatic embryos and ways to store them over longer periods.

At present seedlings at an age of 4 years (2 years in the nursery + 2 years in the field) are sold at approximately 0.5 euro each. Due to better quality of cloned material, the SE produced plants may not need to meet this price, but production

costs must be reduced from the present state in the research laboratory. The existing production is very labor intensive, and automation of specific processes will be an efficient way of reducing costs. For nordmanns fir the two most 'labor expensive' processes are: 1) selection of mature embryos and transfer from maturation medium to germination medium, and 2) transfer of germinated and rooted plants from sterile conditions to soil (Find et al 2009). A previous Danish project aimed at developing automated solutions for these two processes. The conclusion of this work was that it was possible to develop automated handling for the two described processes with a handling time of approximately 4 sec per plant. The most challenging point to establish an automated set up was not the technical aspects or image analysis, but to ensure the required control and synchronization of the biological processes (Find et al 2009).

Integration of the SE production of plants into the existing production of seedlings has been an important objective. The impression was that this was the only way of reducing the additional costs related to the prolonged nursery culture. However, experience in recent years has shown that it is possible to increase growth rate of SE produced plants considerably by intensive management in the nursery. SE produced plants are ready for transfer to the field after 2-3 years of growth, whereas seedlings in the existing production are transferred after 4 years. For this reason it is probably more cost effective to intensify the nursery production of SE plants and to take advantage of the uniformity of clonal propagated plants to optimize growth parameters for each specific clone.

In addition to being a very promising method for enhancing gains from tree-breeding programs and for bulk propagation of elite trees, the SE system offers an excellent basis for development of new methods for future breeding programs. For nordmanns fir the aim is development of protoplast cultures for somatic hybridisation, artificial seeds for improved storage and introduction of new traits by genetic engineering (Find et al 2005).

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